Virulence of Indigenous Isolates of *Metarhizium anisopliae* to Life Stages of the Coconut Rhinoceros Beetle (Coleoptera: Scarabaeidae: Dynastinae) in Papua New Guinea

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Abstract: Coconut, *Cocos nucifera* L., is important crop in PNG and has been challenged with the invasion of pests. The coconut rhinoceros beetle (CRB), *Oryctes rhinoceros* is a serious pest to coconut and threatens the oil palm industry. In recent years, CRB-G biotype evaded the existing biological control agent *Oryctes nudivirus* introduced into the Pacific Islands, in the 1960s. Using pheromone trapping and visual rating, this study assessed population and damage by CRB in Markham Valley, Morobe province; and investigated the virulence of two indigenous isolates of *Metarhizium anisopliae* on CRB life stages by topical inoculation under laboratory conditions. Moderate damage levels was recorded on coconut palms in Daeng villages. Mixed sex CRB were caught in the 3 seasons of trapping, with more males trapped than females at the sites. Laboratory tests revealed that the two isolates varied in germination, time to sporulation on cadaver and virulence. Larval and adult CRB survival decreased by 20% through time when inoculated with the spores of *M. anisopliae*. At 21 and 25 DAI, isolate Ma2 caused high mortality at the shortest time on the larval (82%) and adult (80%), respectively. Isolate Ma2 has the potential as a microbial control agent against CRB. Further evaluation is required for high spore dosage application, spore transmissibility, and 'lure-and-kill' strategy under field conditions.

Keywords: *Oryctes rhinoceros*, *Metarhizium anisopliae*, coconut, pheromone trapping, Papua New Guinea

1. INTRODUCTION

Coconut (*Cocos nucifera* L.) is a subsistence crop, commonly grown around the coastal region of Papua New Guinea (PNG). Yet it is an old industry in PNG that once contributed greatly to the country's economy and subsequent growth and development (Hanson et al, 1998). However, copra production declined over the years due to several factors including fall in global prices that led to abandoning of plantations and infestations by insect pests and diseases, including rhinoceros beetles (KIK Report, 2016). Population of the coconut rhinoceros beetle (CRB), *Oryctes rhinoceros*, had been detected in Guam in 2009 (Marshall et al, 2017). This CRB population was reported to have evaded the biological control agent, *Oryctes nudivirus* (*OrV*); a virus introduced in the 1980s into the Pacific islands, including PNG. Due to its evasion, molecular studies by Marshall et al (2017) had led to the discovery of a biotype; referred to as the CRB Guam biotype (CRB-G). This biotype was detected in PNG in 2010-2012 National CRB Survey (Ero et al, 2016). The development of resistance by CRB-G to *OrV* maybe possibly due to sole reliance of a single biocontrol agent in the wild, and modifications of female oviposition behaviour (Manlet et al, 2018). CRB-G, unlike the Samoan biotype (CRB-S) successfully suppressed by *OrV*, is the most destructive pest to coconut and poses a threat to the oil palm industry (Ero et al, 2016; Jackson et al, 2020). Coconut rhinoceros beetle infestations on coconuts results in up to 50% palm mortality and significant losses in some islands in the Pacific including Samoa and PNG (Jackson et al, 2020), however, these recorded losses may have increased in recent times. In PNG, neglected coconut gardens in the vicinity of commercial oil

palm plantations may act as CRB reservoir for future infestations on oil palm (*Elaeis guineensis* L.), as it is evident in recent times that CRB is slowly but steadily becoming an oil palm pest as well (PNGOPRA, 2014). Heavy infestations on coconut and oil palm have profound effects on rural communities that depend entirely on these crops because they are the major income generators for these coastal communities.

Entomopathogenic fungi (EPF) such as *Metarhizium* spp. are naturally occurring fungi successfully utilized as biological control agents against insect pests and plant disease antagonists (Ramle et al, 2009; Rajula et al, 2021). *Metarhizium* is naturally associated with CRB and related dynastid beetles (Bedford, 1980; 2013) and was first discovered to infect larvae and adult CRB in breeding sites in coconut and oil palm debris. Due to this natural association between the fungus and CRB and failure of *OrV* to regulate wild populations of CRB in general, the search for indigenous strains of *Metarhizium* is essential for their utilization as potential biological control agents. Indigenous strains of EPF are more promising for the management of indigenous pest species, which are proven effective on CRB as in Malaysia and the Solomon Islands (Ramle et al, 2007; 2009; 2011). At present, there is limited knowledge into using *Metarhizium* and other EPF for CRB control although related species of scarabs have been reportedly susceptible to this entomopathogenic fungus (Theunis & Aloal'i, 1998; Moxon, 2008). This study was conducted to assess the CRB damage levels on coconuts and oil palm in the Markham Valley and investigate the efficacy of a strain of commercially-formulated *M. anisopliae* and indigenous strains of *M. anisopliae* on life stages of CRB.

2. MATERIALS AND METHODS

2.1 CRB infestation/damage and population surveys

This exercise was carried out to determine the levels of CRB infestations, purposely for trapping and collection of adult CRB for laboratory studies. Between 2018 and 2019, field observations were conducted in the Markham Valley for typical V-shaped cuts on fronds of coconut palms following assessments by Ero et al (2016). Subsequently, local farmers and field officers of Markham Farms Ltd were interviewed regarding damage of palms by the CRB on coconut and oil palm, respectively. Trapping devices were stationed at 100-150 m apart in Daeng village (GPS; 6°23′09″S, 146°18′23″E), Mutzing, Markham District. Pheromone trap is produced by male CRBs and is a common control technique in Malaysian oil palm plantations (Manjer et al., 2014; Kamarudin et al., 2007) and is now being incorporated into the control measures of CRB in the Pacific including PNG.

Pheromone lure (ethyl-4-methyl loctanoate P046-Lure, ChemTica Internacional, Costa Rica) was incorporated into a trapping device, consisting of 15 cm Ø × 3 m PVC pipe and a collection bucket at the bottom following Ero et al (2016) with modifications. Two 30 cm^2 holes cut out on opposite sides of the pipe just below the edge of the pipe and the holes were not parallel to each other but 10 cm apart (Fig 1). Tie wire was wired across the diameter right above the cut-out holes and a lure sachet was suspended. This method was adopted because it is inexpensive with good precision. Further field surveys were conducted in the village in a mixed crop field (56,750.88 m^2) containing over ninety coconut palm trees planted in no specific order, with understory occupied by 60 cocoa trees, some banana trees and shade trees. The type of defoliation observed gave the basis for infestation level and it was labeled accordingly as no defoliation, light defoliation (1-10% damage), moderate (11-30%) and severe (> 30%).

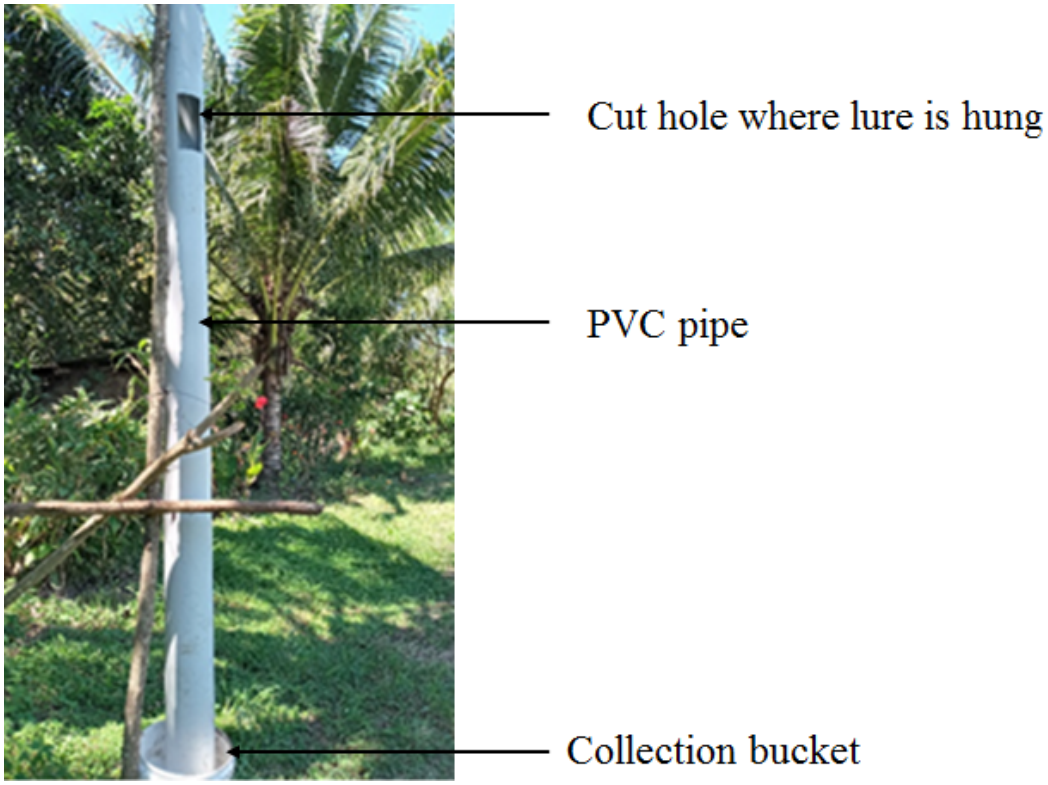

Figure 1. CRB lure setup with PVC and collecton bucket. CRB attracted to the lure fly into the cut holes on each side and fall through the PVC pipe into the bucket.

A rain gauge was also stationed in the Daeng Village to determine rainfall at the course of the CRB trapping. Traps were monitored every 5 days for 7 months. Caught adult CRB in the collection bucket were first counted then sexed using developed keys for distinguishing sexes (Bedford, 2013), placed in clear plastic cages and brought to the laboratory. Additionally, dead but standing coconut palms were felled and searched for incidence of CRB larvae.

2.2 Test insects rearing

CRB larvae (1^{st} – 2^{nd} instar) were field-collected in debris heaps and rotten tree stumps, and reared in captivity to 3^{rd} or 4^{th} instar. Larvae were separated from other scarab species larvae using identification keys (Beaudoin-Ollivier et al, 2000). A mixture of cattle dung and sawdust at a 1:2 ratio was added into each rearing cage (39 cm × 30 cm × 25.5 cm, L×W×H) to 15 cm depth. Adult CRB beetles were caught from the wild using a pheromone previously described. The insects were brought to the laboratory and reared in captivity in 15 L clear plastic boxes, 4 beetles (mixed sex) to a box. Each box had dices of ripe Cavendish banana and apple as food source and replaced once every 5 days (Bedford, 2014). Rearing cages were kept at room temperature (26 ± 2 ºC) in the laboratory, Unitech Biotechnology Centre.

2.3 Source of fungi

The details of *Metarhizium* strains used in this study are provided in Table 1. Isolates Ma1 and Ma2 were identified as *M. anisopliae* using ITS primers (Gurr et al, 2022). Spores from glycerin-preserved agar slants of the isolates were cultured either on fresh Sabouraud dextrose agar with 5% yeast (SDAY) or potato dextrose agar (PDA) media plates to facilitate fungal growth. A commercial-formulated strain of *M. anisopliae* (Oryx-X®, FGV Agri Services, Malaysia) used for CRB control in Malaysia, was donated by Dr Mark Ero (PNGOPRA, PNG) for comparison against the indigenous isolates; however, due to germination and growth failure in culture plates after many attempts, it was not included in this study.

Table 1. Source of *Metarhizium anisopliae* isolates tested against CRB life stages.

Isolate	Source	Locality	Elevation
Ma1	Baited from soil samples collected from sweetpotato gardens	Ruinga, Jiwaka province,	5.8691° S, 144.6973° E, 1602 m asl
Ma2	Baited from soil samples collected from sweetpotato gardens	Asaro Valley, Eastern Highlands province	6.5862° S, 145.6690° E, 1570 m asl

2.4 Laboratory studies

Experiment 1: CRB larval bioassay

A spore suspension was prepared in 0.5% Tween 20® solution to challenge CRB larvae. Conidia were harvested from 16-day old cultures; spore counts and dilution was conducted with Neubauer haemocytometer and concentrations adjusted to 5×10^8 conidia/ml. Five to 6 CRB larvae were inoculated by immersing larva individually into the spore suspension for 2-3 s (Ondiaka et al, 2008; Dotaona et al, 2015). Inoculated larva was placed on a paper tissue for 30-60 s to remove excess suspension on the larva. Only 3rd to 4th instar larvae were used in the study. Larvae for the control treatment were immersed in 0.5% Tween 20® solution. Inoculated larvae were placed individually in disposable clear plastic lunch boxes (14 cm × 11 cm × 6 cm), containing a mixture of cow dung-sawdust as a food source. Altogether there were 3 treatments replicated 6 times in a completely randomized design. Observations were made on 7, 14 and 21 days after inoculation (DAI). Larvae that were killed (or cadaver) at 7, 14 and 21 DAI were removed from the treatment containers and were lightly rinsed with sterile distilled water (dH_2O), soaked in 0.05% NaOCl and a 60 s soak in 70% ethanol for surface sterilisation. Sterilized cadavers were finally rinsed with dH_2O three times then air dried on paper towels. A moistened Whatman® No. 1 filter paper (80 mm Ø) was placed into a 250 ml white foam cup and cadaver placed on it. Cups containing cadavers were sealed and incubated at dark at room temperature (26 ± 2°C) to allow mycosis and sporulation.

Experiment 2: Adult CRB bioassay

Bioassays on wild-collected CRB were conducted between June and August 2023. Adult CRB were trapped in Markham Valley using the previously described trapping procedure. At the bioassay, adult CRB were separated from the rearing boxes and starved for 24 h. Similar bioassay procedures as in Experiment 1 were followed. Spore viability test was carried out for the two *Metarhizium* isolates prior to preparation of the spore suspensions. Adult CRB beetle was inoculated by immersing the insect into a spore suspension for 3-4 s. Control beetles were immersed in 0.05% Triton X-100 solution. Ten beetles were inoculated per replicate, and each *Metarhizium* isolate was replicated 4 times. Inoculated beetle was placed on a tissue paper for 60 s to absorb excess water and placed in disposable clear plastic lunch box (14 cm × 11 cm × 6 cm) with cut lid (2 cm × 1 cm opening) for ventilation. The lunch box contained a 50 g diced ripe banana, was subsequently replaced after 3 days by a 50 g diced apple. Boxes were placed in the dark at room temperature (26 ± 2°C) and positions changed every 3 days. Beetle mortality was recorded daily. Beetles motionless and showing stiff legs were considered as dead, removed from the rearing box and placed on moistened filter paper in a 250 ml foam cup, sealed and incubated to encourage mycosis and sporulation. Time to fungal mycosis on dead beetle was also recorded. The bioassay was repeated after two months.

Data analysis

Data on CRB infestation survey and the laboratory bioassays were first collated in Microsoft Excel (2010). Percent damage was calculated by the number of coconut palms showing symptoms of CRB infestation over the total number of coconuts surveyed.

For the CRB trapping, the number of beetles from monthly traps in each year and sex ratios were square root transformed [$\sqrt{(x + 0.5)}$] and inverse sine transformed, respectively, to achieve approximate normality (Zar, 1974; Wilson & Hardy, 2002). Sex ratio data were pooled by month and one-way analysis of variance (ANOVA) performed. A two-way ANOVA was used to separate the effects of months and seasons (years) on beetle catches.

For the analysis of spore germination data, one-way ANOVA was used. Mann-Whitney test (Zar, 1974), a non-parametric test, was used to analyze time to sporulation.

For experiments 1 and 2, the data on repeated bioassays were combined as no significant interactions were detected between effects of treatment and the repeat times. If ANOVA indicated a significance *F* value, Fisher's least significance difference (LSD) was used to compare treatment means at 5% significance level. Beetle catches (sex ratios) and spore germination data analyses were performed in SPSS for Windows (Version 26.0, Armonk, NY) and bioassay data analyses performed in Genstat 14th Edition (VSN International Ltd., UK).

3. RESULTS

3.1 CRB infestation survey and trapping

Ninety sampled palms of the total 120 coconut palms showed 30% of the trees have fronds with V-shaped damage typical of CRB feeding, and 20% of the physically assessed trees that were simultaneously observed when assessing damage on fronds have both old and new boreholes at the frond base. No CRB was reported in the oil palm plantations, however, infestations were observed mainly on coconut palms at the vicinity of oil palm blocks. The coconut palms in Daeng village were subsequently classified as moderately damaged (15%).

The pheromone traps attracted mixed sex beetles with significantly high beetle catches ($F_{6,12} = 3.527$, $P < 0.05$) in June-July during the 3 years (Fig 2A). Beetle catches between seasons were not statistically different ($F_{2,12} = 0.157$, $P = 0.857$), however, high proportions of up to 80% ($F_{1,12} = 9.05$, $P < 0.05$) male CRB were caught at the trapping period. As observed in this time of the year, minimal rainfalls (Fig 2B) were received, up to a monthly average of 47 mm.

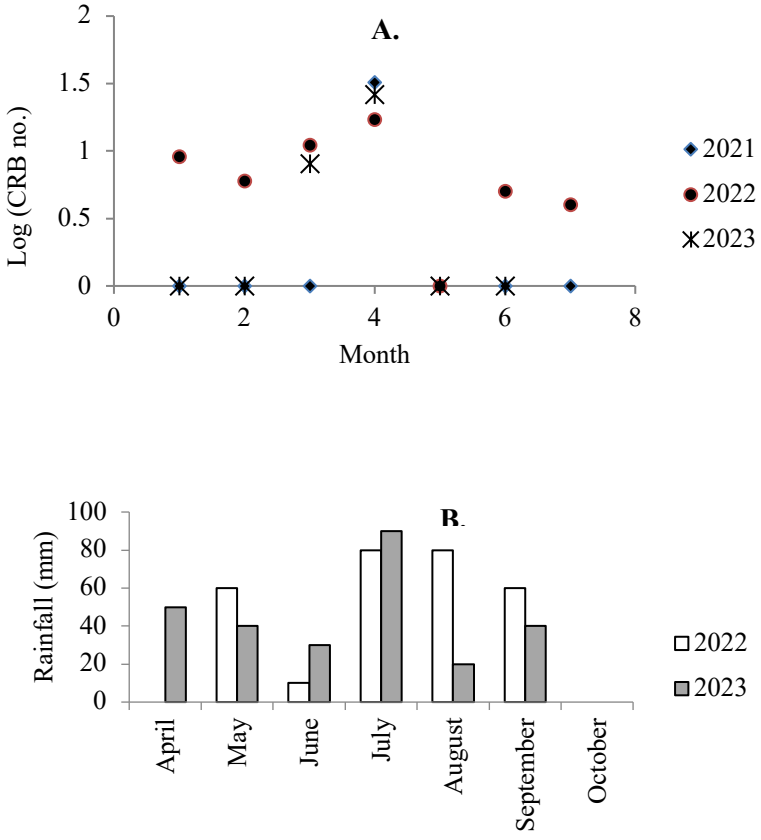

Figure 2. CRB adult catches (A) using aggregate pheromone and, (B) rainfall received in two years in experimental site, Daeng village.

Average catch and rainfall in 3 years was regressed to determine if any relationship exists. Regression showed a statistically significant relationship between the rainfall and beetle catch ($R^2 = 0.67$, $F_{1,5} = 10.08$, $P < 0.05$) (Fig 3).

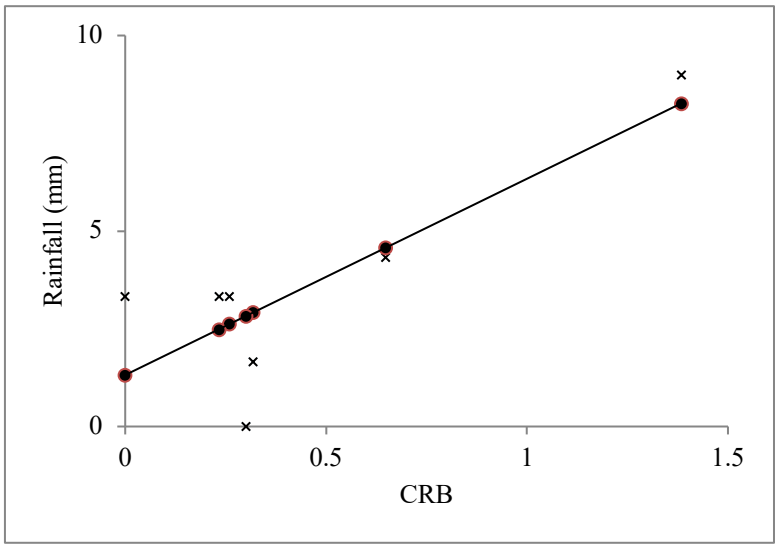

Figure 3. Relationship between rainfall and CRB catch with regression best fit line (Y = 5.02*x* + 1.317).

3.2 Laboratory studies

Laboratory bioassays to test the virulence of indigenous *M. anisopliae* on different life stages of CRB are presented Figures 4 and 5. Inoculated CRB larvae and adult with *M. anisopliae* isolates reduced mean survival. In the larval stage, death was initiated before 5 DAI for the experimental treatments and after 10 DAI for the control treatment (Fig 4). The median lethal time (LT_{50}) for the two isolates at conidia dosage 5×10^8 conidia/ml was reached between 18 and 19 DAI.

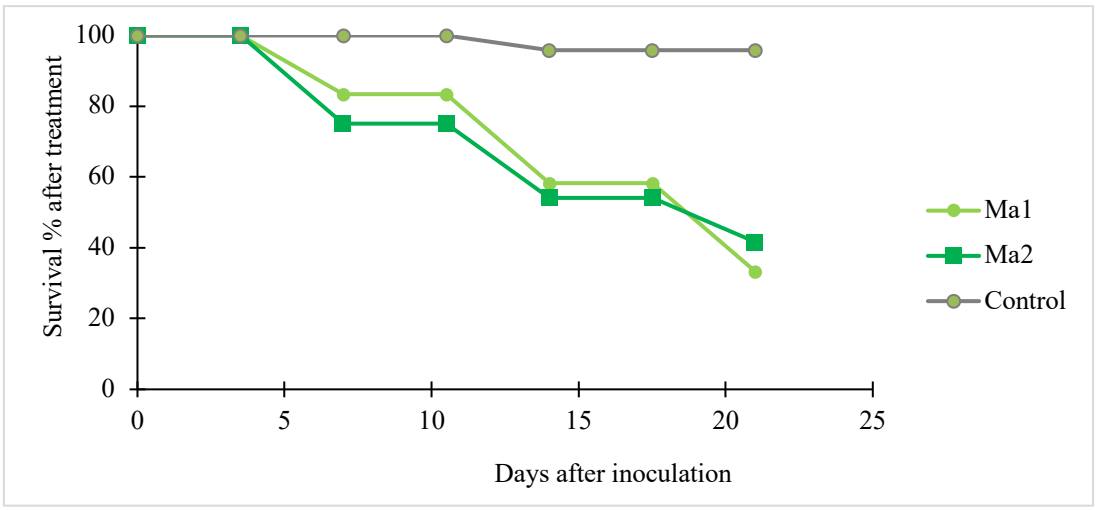

Figure 4. Mean survival times of CRB larvae (3rd/4th instar) over time after inoculation with isolates of *M. anisopliae*.

Despite having similar spore germination, rapid sporulation of isolate Ma1 was observed on the larval cadavers, compared to isolate Ma2 (Table 2). At 21 DAI, isolate Ma2 caused the highest larval mortality (Table 2). The mean survival in inoculated adult CRB likewise declined over the 25-day exposure, compared to the control treatment. Beetle deaths occurred at 7 DAI in inoculated adult CRB and LT_{50} for isolate Ma2 was reached at 17 DAI (Fig 5). Time to sporulation on the incubated beetle cadaver was rapid in isolate Ma1, taking 6 days, than in Ma2. However, after 25-day exposure, Ma2 of the two isolates caused the highest beetle mortality (Table 3).

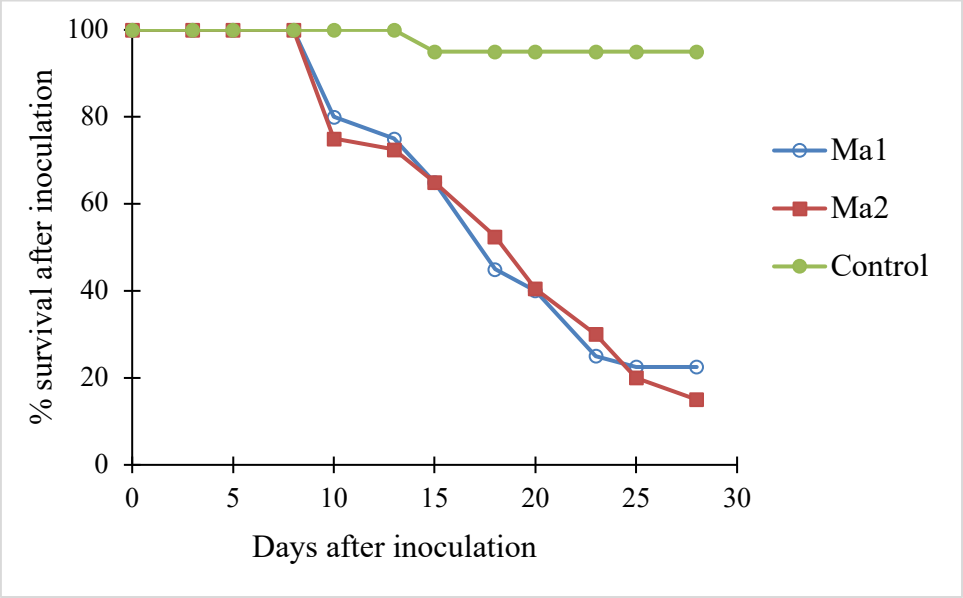

Figure 5. Mean survival times of adult CRB over time after inoculation with isolates of *M. anisopliae*.

Table 2. Corrected mortality of larvae CRB after inoculation with *Metarhizium anisopliae* isolates and incubation at 26±2°C. DAI, days after inoculation. *n* = 10 larvae per replicate.

Isolate/treatment	% spore germination	Time (days) to sporulation	Corrected % mortality		
			7 DAI	14 DAI	21 DAI
Ma1	80 ± 2a*	5.5 ± 1.1a[1]	17a†	45a†	70a†
Ma2	86 ± 3a	7.4 ± 1.2b	20a	50b	82b
Control	-	-	0b	6c	9c

*Means with the same letter within a column do not differ significantly at $P > 0.05$ (ANOVA). [1]Means with the same letter within a column do not differ significantly at $P < 0.05$ (Mann-Whitney test). †Means with the same letter within a column do not differ significantly at $P < 0.05$ (ANOVA, LSD test).

Table 3. Corrected mortality of adult CRB after inoculation with *Metarhizium anisopliae* isolates and incubation at 26±2°C. DAI, days after inoculation. *n* = 10 beetles per replicate.

Isolate/treatment	% spore germination	Time (days) to sporulation	Corrected % mortality	
			15 DAI	25 DAI
Ma1	78 ± 4a*	6.2 ± 0.1a†	50a[1]	74a[1]
Ma2	89 ± 3b	7.5 ± 0.5b	39b	80b
Control	-	-	0c	5c

*Means with the same letter within a column do not differ significantly at $P > 0.05$. †Means with the same letter within a column do not differ significantly at $P < 0.05$ (Mann-Whitney test). [1]Means with the same letter within a column do not differ significantly at $P < 0.05$ (ANOVA, LSD test).

4. DISCUSSION

CRB is an invasive insect to cultivated and ornamental palms, and severe infestations have been reported elsewhere in PNG. In our survey in the Daeng village, Markham Valley, symptoms of CRB infestations were similar to descriptions reported in Port Moresby and offshore islands including New Britain and New Ireland (Ero et al, 2016). Moderate infestations have been recorded on coconut palms but rarely on oil palm. One reason for rare infestations on oil palm was due to the fact that Markham Farms practiced crop sanitation by removing old stumps which attract CRB breeding, and regular monitoring by using pheromone lures incorporated with insecticide to lure and kill. Early larval instars (1^{st} – 2^{nd} instar) were recovered from rotting felled coconut palms, within the trunk of dead standing palms and beetle-infested young palms (2-3 years old). It is evident that female CRB oviposits within the bark of dead standing trunk, which is rarely reported. Since the traps had been incorporated with aggregation pheromone, mixed sex beetles were caught; however, we observed higher male:female ratio compared to other studies reporting lower male:female sex ratio (Oehlschlager, 2007; Manjeri et al, 2014). Several studies (e.g. Kamarudin & Wahid, 2004; Aidoo et al, 2022) reported monthly rainfall above 20 mm to stimulate the flight movement of CRB in search for breeding sites, hence increase in trapped beetles. Our findings showed that in areas where regular dry spells occur, as is the case of Markham Valley, prolonged dry spells followed by minimal rains undoubtedly encouraged CRB flight movement. Prolonged wet seasons, on the other hand, are unfavourable to CRB survival, as these cause high beetle drowning at mating and female oviposition, thus high mortality (L. Kuniata, *pers. comm*., Ramu Agri Industries).

The two *M. anisopliae* isolates tested here were sourced from the soil with histories of sweetpotato cultivation in the highlands of PNG, and these isolates were highly virulent to sweetpotato weevils (*Cylas formicarius* and *Euscepes postfasciatus*, Coleoptera) in previous studies (Gurr et al, 2022). In many cases, *M. anisopliae* isolates maintained considerable spore viability, virulence and host specificity to insect species of the same order or of the same genus,. For instance, the isolate of *M. anisopliae* sourced from adult taro beetle (*Papuana woodlarkiana*) in PNG exhibited high virulence to another species of taro beetle (*P. uninodis*) in Fiji (Theunis & Aloali'i, 1998)), and *M. lepidiotae* that infects *Lepidiota* beetles (Coleoptera) and other scarab species in Australia and PNG (Driver et al, 2000; Bischoff et al, 2009). Certain isolates may have low host specificity as those reported to cause high mortality on insect species of different orders; fruit flies (Diptera) (Dimbi et al, 2003) as well as sweetpotato weevil (Coleoptera) (Ondiaka et al, 2008). Our study showed that one of the two *M. anisopliae* isolates (Ma2) was more virulent, however, the two isolates exhibited some host specificity on Coleoptera species at this stage. The isolates varied significantly in spore germination, virulence and time to sporulation on cadaver. The relationship of age of wild-caught CRB and sex type to fungal infection were not established in this study. While isolate Ma1 exhibited high % germination rate and fast sporulation on cadaver, which are desirable traits for fungal transmission and possible natural epizootics, it caused low mortality on CRB larval and adult stage in comparison to isolate Ma2. We were unable to test their efficacy at multi-dose spore concentration and transmission studies from infected to healthy individuals (Dotaona et al, 2015; Khun et al, 2020). The two isolates are unique; sourced from the high altitudes and are virulent on an insect host in the lowlands of PNG, hence may be considered as having altitudinal plasticity. Future field studies will require testing of higher spore concentrations of isolate Ma2, its efficacy and spore transmissibility, and 'lure-and-kill' strategy by incorporating Ma2 infective spores in the trapping system.

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